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CHARACTERIZATION OF LIPOPROTEINS ISOLATED FROM THE HEMOLYMPH OF THE SPIDER *LATRODECTUS MIRABILIS* (ARANEAE, THERIDIIDAE)

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ABSTRACT. Two high density lipoprotein fractions (HDL_1 and HDL_2) were isolated from the hemolymphatic plasma of the spider *Latrodectus mirabilis* (Holmberg 1876). For each, the hydrated density, the electrophoresis mobility of the apoproteins, and the lipid classes composition were determined. The HDL_1 fraction carried 80% of the total plasma lipids, which were predominantly composed of phospholipids, free fatty acids, and triacylglycerols. The apoprotein composition of this fraction showed two main bands of 90 and 103 kDa. The HDL_2 fraction was composed primarily of phospholipids, free fatty acids and cholesterol. This fraction contained hemocyanin as the principal apoprotein. When the HDL_2 fraction was separated into three subfractions, all of them contained hemocyanin, with the main subfraction containing the hexameric form of the respiratory pigment. With regard to triacylglycerol transport, lipid and apoprotein compositions and hemocyanin role in the lipid transport, these lipoproteins (HDL_1, HDL_2) show similarities and differences when compared to the two spider species already studied.

Keywords: Lipoproteins, *Latrodectus*, hemolymph

Lipids can not circulate freely in an aqueous medium due to their hydrophobicity. Notwithstanding, they are transported by hemolymph from the sites of uptake or synthesis to the sites of storage and usage via water-soluble lipoproteins. Lipid circulation systems in invertebrates have been studied only in the phyla Arthropoda and Mollusca. Although the mechanisms of lipid circulation are well-known in arthropods such as insects and crustaceans, in arachnids there is little available information on plasma lipoproteins. Lipoproteins of high density have been detected in the hemolymph of spiders, scorpions, solpugids, and mites. According to their apoprotein components, lipoproteins in spiders, scorpions, and solpugids showed similar characteristics to those of insect lipophorins (Haunerland & Bowers 1989). In spiders, the lipoprotein lipid composition was extensively studied by Haunerland & Bowers (1987) in *Eurypelma californicum* (Ausserer 1871) (Theraphosidae) and *Polybetes pythagoricus* (Holmberg 1874) (Heteropodidae) by Cunningham et al. (1994). In *E. californicum*, the high content of diacylglycerols and phospholipids also resembles the composition of insect lipophorins. In *P. pythagoricus*, three plasma lipoproteins were detected and characterized. One of them was of high density and evidenced similar apoproteins to the ones called lipophorins. In contrast, its lipid composition was rather different, containing a large amount of phospholipids and triacylglycerols. The other two lipoprotein fractions of *P. pythagoricus*, of high and very high density, also contained phospholipids and triacylglycerols as major lipids; but hemocyanin was the predominant apolipoprotein (Cunningham & Pollero 1996).

The differences found in the lipid and apoprotein compositions of the two previously studied spiders led us to investigate the lipoproteins in a third species, *Latrodectus mirabilis* (Theridiidae), a widely distributed species. The literature reports studies on the biology and ecology (González 1981; Estévez et al. 1984) and venom components (Flo et al. 1991). There is no available study on the biochemical and physiological aspects of the lipid circulation. This study describes the composition of the lipid and protein moities of two plasma lipoproteins isolated from the *L. mirabilis* hemolymph. The role of triacylglycerols as circulating energetic lipids, of hemocyanin

as apolipoprotein, as well as composition similarities and differences between these lipoproteins and those of other spider species, are discussed.

METHODS

Hemolymph collection and lipoprotein separation.—We collected adult females of *Latrodectus mirabilis* (deposited in the Museum of Natural Sciences, La Plata) in summer from the hills of Sierra de la Ventana, province of Buenos Aires, Argentina. After the legs were severed from the body, the spiders were placed in tubes and centrifuged at low speed in order to obtain hemolymph.

Plasma was centrifuged in a gradient density on 3 ml NaBr −1.21 g/ml, with Trasylol as protease inhibitor, at 178,000 G for 22 hours in a Beckman L8 70M centrifuge with a SW60 Ti rotor. As the density of the spider plasma was 1.006 g/ml, a saline solution of the same density was run simultaneously as blank. The total volume of the tubes was fractionated from top to bottom into 0.3 ml fractions. The density and total proteins in each fraction were monitored by refractometry and light absorption at 280 nm, respectively.

Lipid extraction and analysis.—Total lipids from the lipoprotein fractions were extracted with chloroform/methanol (Bligh & Dyer 1959). Total lipids were analyzed on Merck high performance thin-layer chromatography (HP-TLC) plates. Hydrocarbons were separated from other neutral lipids by development in hexane-benzene (70:30 v/v). Polar lipids were resolved by developing the plates in chloroform/methanol/acetic acid/water (65:25:4:4 v/v) and hexane/diethyl ether/acetic acid (80:20:1.5 v/v) for neutral lipids. Appropriate standards were used. Spots were visualized with I2 vapors and identified by comparison with known standards.

The quantitative determination of the lipid classes was performed using a thin-layer chromatograph coupled to a flame ionization detector (TLC-FID) system. A full description of this technique was given by Ackman (1990). FID scans were performed on an Iatroscan TH-10 analyzer (Iatron Laboratories, Japan). The development solvent systems used were: hexane/benzene (70:30 v/v), benzene/chloroform/formic acid (70:25:2 v/v) and chloroform/methanol/water (70:25:3 v/v). Lipid classes were quantified by comparison with known amounts of standards run under the same conditions and using monoacylglycerol as internal standard. Total lipids were calculated by the summation of individual lipid weights.

Gel permeation chromatography.—A very high density lipoprotein fraction separated from the gradient was analyzed under native conditions by preparative high-pressure liquid chromatography on a Superdex 200 HR 10/30 column (Pharmacia, Uppsala, Sweden) using 0.1 M Tris-HCl (pH 8.0), containing 10 mM $CaCl_2$ and $MgCl_2$, at the flow rate of 0.4 ml/min. Proteins were detected at 280 nm. Lipoprotein subfractions were eluted. The column was calibrated for molecular weight using thyroglobulin, ferritin, catalase, bovine serum albumin (BSA) and ribonuclease A (Pharmacia, Sweden) as protein markers.

Characterization of apoproteins.—Total protein concentration in each fraction isolated from the density gradient was measured colorimetrically (Lowry et al. 1951). These fractions and subfractions isolated by HPLC, were extensively dialyzed against 10 mM Tris-HCl (pH 6.8) and analyzed by electrophoresis under dissociating and native conditions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in both, 8% continuous slab gels overlayered with 4% stacking gels and in gradients of 4–20% gels (Laemmli 1970) in a mini-slab electrophoresis unit (8 × 10 cm). The resolving gel buffer was 0.375 M Tris-HCl and the stacking gel buffer was 0.125 M Tris-HCl. The electrode buffer contained Tris-glycine 0.025 M Tris, 0.192 M glycine (pH 8.3). Proteins were visualized by staining with Coomassie Brilliant Blue. Molecular weight standards (HMW, Pharmacia, Uppsala, Sweden and Markerkit, Sigma Chemical Co., St. Louis, Missouri) were run in parallel lines.

The presence of hemocyanin in the fractions was monitored by spectrophotometric scans from 200–700 nm, before and after sample treatment with 0.2 M KCN solution (Nickerson & Van Holde 1971). A DW-2000 UV-Vis spectrophotometer SLM Aminco was used.

RESULTS

Isolation of plasma lipoprotein fractions.—After plasma centrifugation in density gradients, two colored bands appeared: a

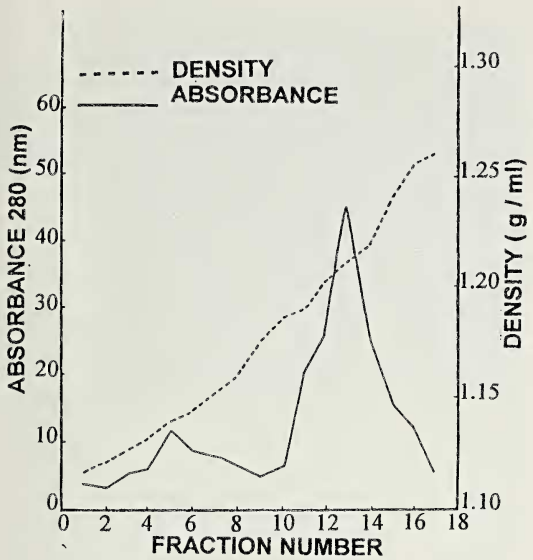

Figure 1.—Total protein (absorbance at 280 nm) and density distribution in plasma fractions of *Latrodectus mirabilis*. Plasma was centrifuged in a NaBr gradient and fractionated.

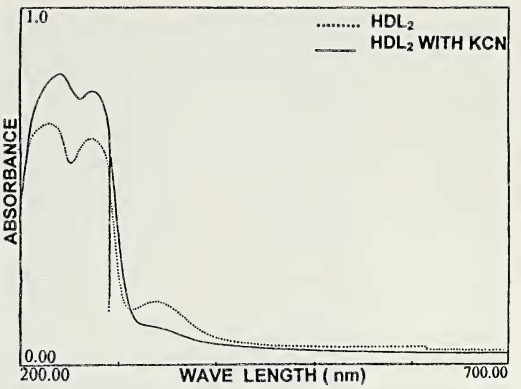

Figure 2.—Hemocyanin characterization. Spectrophotometric scans of hemocyanin from HDL_2, before and after sample treatment with KCN. Broken line = without KCN; solid line = with KCN.

brownish one (HDL_1) and a grey one (HDL_2), whose densities were 1.13 g/ml and 1.19–1.20 g/ml, respectively. Measurements of absorbance at 280 nm performed in each fraction from gradients showed a protein profile with two maxima, one of them (the smallest) corresponded to HDL_1 and the major one to HDL_2 (Fig. 1). Plasma fractions out of colored bands showed relatively low protein and no lipid concentrations. Both colored fractions were isolated and characterized separately.

Hemocyanin was present in the HDL_2 fraction. The respiratory pigment was identified by modification of its characteristic absorption spectrum; the absorption band of 340 nm disappeared when samples were treated with KCN solution (Fig. 2).

Lipid and protein characterization of HDL_1.—The HDL_1 carries 80.4% of the total plasma lipids. Lipids in this fraction were analyzed in their component classes. Phosphatidylcholine, phosphatidylethanolamine, free fatty acids, triacylglycerols, cholesterol and hydrocarbons were identified qualitatively using HP-TLC.

The quantitative lipid composition, determined by TLC-FID, is shown in Table 1. The predominant lipids were phospholipids (35%) and free fatty acids (33%). Triacylglycerols

Table 1.—Composition of HDL_1 and HDL_2 isolated from plasma of *Latrodectus mirabilis*. The lipoproteins were isolated by ultracentrifugation in density gradient. Lipids were identified after separation by HP-TLC and quantified by TLC-FID. Proteins were measured by colorimetry. Results are the average of three determinations (100 animals) ± SD. Data are expressed as weight percent of lipids as determined by TLC-FID.

Component	HDL_1	HDL_2
Lipid classes (percent weight/weight)		
Hydrocarbons	4.0 ± 0.3	14.1 ± 2.3
Triacylglycerols	24.1 ± 0.8	8.3 ± 2.1
Free fatty acids	33.0 ± 1.3	28.4 ± 4.2
Cholesterol	4.2 ± 0.3	20.1 ± 2.7
Diacylglycerols	Traces	Traces
Phosphatidyl ethanolamine	3.6 ± 0.2	4.0 ± 0.5
Phosphatidyl choline	31.1 ± 0.3	25.1 ± 2.8
Total lipids (mg/ml hemolymph)	1.23 (20.3%)	0.3 (1.0%)
Total proteins (mg/ml hemolymph)	4.83 (79.7%)	31.6 (99.0%)

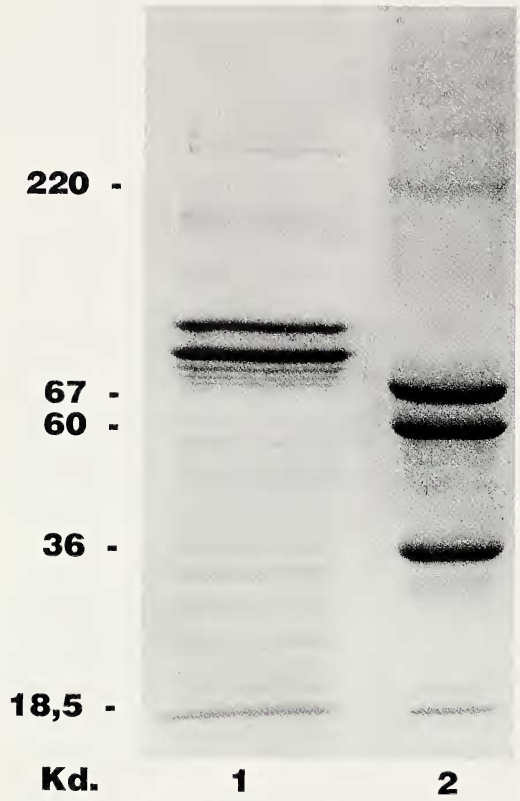

Figure 3.—SDS-PAGE analysis (4–23% acrylamide) of HDL_1 apoproteins from *Latrodectus mirabilis* hemolymph. Kd: Molecular weights of standard proteins expressed in kilodaltons. Lane 1: HDL_1 from *L. mirabilis*. Lane 2: Molecular weight standards (Kd).

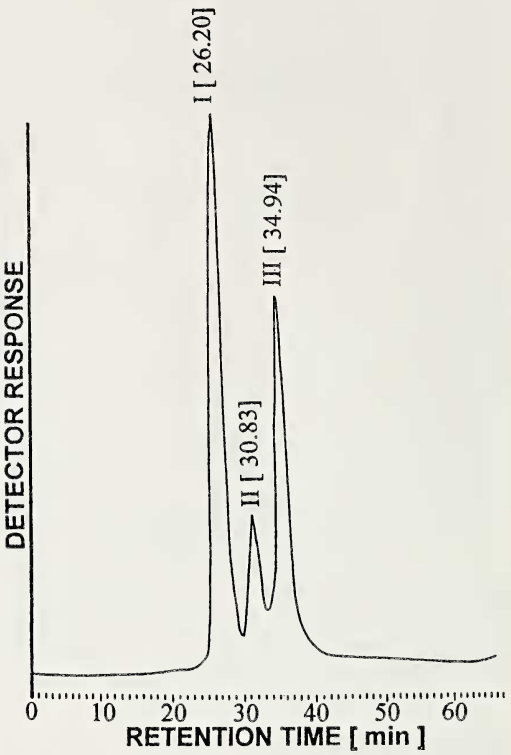

Figure 4.—Elution profile from HPLC of HDL_2 isolated from *L. mirabilis* plasma. Subfractions I, II and III were collected and analyzed separately.

were quite abundant in this fraction whereas hydrocarbons and cholesterol were found in a low proportion. Traces of diacylglycerols were also detected.

Other aliquots of HDL_1 were used to analyze the constituent apoproteins by electrophoresis. Figure 3 shows those results obtained from the electrophoretic analysis performed under dissociating conditions (SDS-PAGE). Among other proteins, two sharp bands of 90 and 103 kDa, respectively, were observed as the major HDL_1 apoproteins.

Lipid and protein characterization of HDL_2.—The HDL_2 lipids were analyzed quantitatively and qualitatively. This lipoprotein fraction carries 19.6% of total plasma lipids. The same lipid classes as those belonging to HDL_1 fraction were identified by HP-TLC and some differences were found in their relative percentages. Phospholipids (29%) and free fatty acids (28%) were the predominant lipids, followed by cholesterol, hydrocarbons and minor quantities of triacylglycerols (Table 1).

Aliquots of HDL_2 were analyzed by HPLC under native conditions using columns of molecular exclusion (Fig. 4). Three subfractions of Mr 440 kDa, 121 kDa and about 70 kDa, respectively, were found. They were eluted from the column, collected and analyzed by electrophoresis under denaturing conditions. Figure 5 (SDS-PAGE) shows two proteins of 76 and 67 kDa, respectively, in the three subfractions isolated from HDL_2.

DISCUSSION

Centrifugation in a density gradient was effective in separating two well-defined bands from *Latrodectus mirabilis* plasma which corresponded to the high density lipoproteins HDL_1 and HDL_2. HDL_1 has a density similar to that of lipophorins isolated from plasma of *Eurypelma californicum* (Haunerland & Bow-

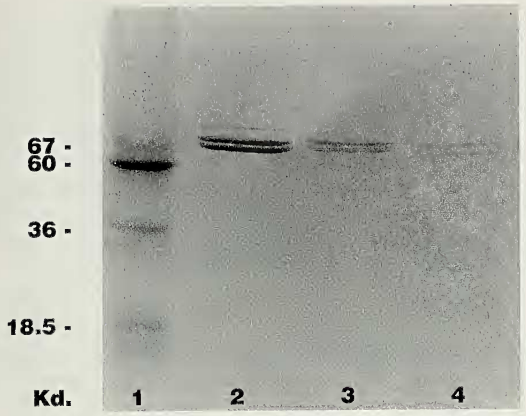

Figure 5.—SDS-PAGE analysis (4–23% acrylamide) of HPLC-fractionated HDL_2 from *Latrodectus mirabilis* hemolymph. Kd: Molecular weights of standard proteins expressed in kilodaltons. Lane 1: Molecular weight standards (Kd). Lane 2: HDL_2 subfraction I. Lane 3: HDL_2 subfraction II. Lane 4: HDL_2 subfraction III.

ers 1987) and of *Polybetes pythagoricus* (Cunningham et al. 1994), which are the only arachnids which have been studied in detail. Its density is also similar to that of lipophorins found in insects (Chino et al. 1981). The HDL_2, though its density is greater than the HDL_1 fraction, is also a high density lipoprotein, and so it can be compared to the HDL previously isolated from plasma of *P. pythagoricus* (Cunningham & Pollero 1996).

HDL_1 is the main lipid carrier fraction in *L. mirabilis* hemolymph since more than three-fourths of the total plasma lipids are associated to it. This quantitative importance in lipid transport locates it at the same level as that of *E. californicum* and above *P. pythagoricus* lipophorin which only carries about 30% of circulating lipids. In contrast, when lipid classes found in this lipoprotein fraction are compared, similarities to *P. pythagoricus* and differences to *E. californicum* are evident. Phospholipids and fatty acids are the predominant lipids; and, as in *P. pythagoricus* lipophorin, triacylglycerols are the most abundant neutral lipids. This fact indicates that triacylglycerols together with free fatty acids are the main circulating energetic lipids in this species, in contrast to *E. californicum* and insects where the presence of large amounts of diacylglycerols characterizes the lipophorins.

The protein moiety of HDL_1 is composed of two principal polypeptides with a molecular weight of 90 and 103 kDa, respectively. This also differs when compared to *E. californicum* and *P. pythagoricus* apolipophorins, and to those found in other arachnids whose protein moieties have been studied (Hauner-land & Bowers 1989). In all these cases as well as in insects, lipophorin particles contain apoproteins of 80 and 250 kDa and a total weight of about 500 kDa. In short, due to its composition, the HDL_1 of *L. mirabilis* is significantly different when compared to HDLs of the same density in other invertebrates that are taxonomically close to it. For this reason, we think it shouldn't be named lipophorin.

In *L. mirabilis*, the HDL_2 could play a secondary role in the hemolymph transport of lipids due to the fact that the lipids associated to this lipoprotein are lesser that those ones bound to the HDL_1. Nevertheless, its lipid composition, with relatively high amounts of hydrocarbons and cholesterol, suggests that HDL_2 could be specialized in the transport of these lipid classes. Although this lipoprotein particle differs from the HDL of *P. pythagoricus* not only in the lipid/protein ratio but also in the lipid classes it transports, both of them carry triacylglycerols but no diacylglycerols as the main neutral acylglycerides.

The electrophoretic analysis of HDL_2 under dissociating conditions, shows protein bands with molecular weights similar to the hemocyanin monomers found in other spiders (Schneider et al. 1977; Lamy et al. 1979; Markl 1986). The removal of copper by KCN treatment confirms this identification. Although we tried to stabilize the hemocyanin, it is very likely that, when handling the samples, there would have been some dissociation of HDL_2 native particles; and, consequently, subfractions of different size would have appeared after gel permeation chromatography. The loss of native conformation of hemocyanin could be the result of changes in the pH, the divalent cation concentration during the processing samples, or due to the use of NaBr in the centrifugation procedure (van Holde & Miller 1986; Hepskovits & Villanueva 1986; Herskovits et al. 1991).

Undoubtedly hemocyanin plays an apolipoprotein role since it is part of this lipoprotein particle as a principal protein. This function of hemocyanin in spiders regarding the lipid transport, in addition to its classical role as respiratory pigment, has been recently re-

ported for *P. pythagoricus* plasma (Cunningham & Pollero 1996) where, however, other polypeptides associated with hemocyanin were also found. In this study, the three subfractions isolated from *L. mirabilis* HDL_2 under dissociating conditions only yielded hemocyanin monomers. This corroborates the apoprotein function of hemocyanin in this lipoprotein. This apolipoprotein role of hemocyanin is not a constant in spiders, since no associated lipids could be detected in tarantula hemocyanin. A similar finding has been reported for molluscs where the hemocyanin of the cephalopod *Octopus tehuelchus* transports lipids (Heras & Pollero 1992), while that of the gasteropod *Ampullaria canaliculata* does not (Garin & Pollero 1995).

In *P. pythagoricus* plasma, we have characterized a third lipoprotein of very high density which is the main carrier of circulating lipids, and which contains hemocyanin as the principal apoprotein (Cunningham & Pollero 1996). The existence of a VHDL has also been reported for *Eurypelma californicum* (Haunerland & Bowers 1989). In this case, however, it was a lipoprotein without hemocyanin which played a secondary role in lipid transport. In *L. mirabilis* no particle with VHDL characteristics has been detected.

In brief, *L. mirabilis* contains two plasma lipoproteins; but their lipid and protein compositions share only a few features with the hemolymph lipoproteins already described other spider species. Such differences in number and composition of plasma lipoproteins in taxonomically close organisms make generalization difficult.

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